ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 217, No. 2, September, pp. 674-681, 1982

Effect of Solvents on the Catalytic Activity of Firefly Luciferase[1,2]

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Received March 17, 1982, and in revised form May 4, 1982

Various solvents stimulate the catalytic activity of firefly luciferase, up to sevenfold. Polyvinylpyrrolidone, polyethylene glycols, and nonionic detergents such as Triton X-100 were the most effective stimulators of the enzyme. Both peak light and total light emission were enhanced in the presence of these solvents indicating an increased turnover of the enzyme. The primary effect of the solvents is on the oxidative reaction rather than the activation reaction. All the experimental data support the hypothesis that the presence of solvent promotes the dissociation of the inhibitory product from the enzyme.

Firefly luciferase from *Photinus pyralis* has been extensively studied by many investigators since it was first crystallized by Green and McElroy in 1956 (1). The enzyme catalyzes the oxidative decarboxylation of luciferyl-adenylate as shown in the following reactions:

$$\text{D-LH}_2{}^{5} + \text{ATP} + \text{E} \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{E}\cdot\text{LH}_2\text{AMP} + PP_i \quad [1]$$

$$\text{E}\cdot\text{LH}_2\text{AMP} + \text{O}_2 \rightarrow \text{E}\cdot\text{P} + \text{CO}_2 + \text{AMP} + h\nu \quad [2]$$

The first reaction [1] is the activation of luciferin to form an enzyme bound luciferyl adenylate and PP_i. This reaction is relatively specific for ATP and in order for reaction [2] to proceed D-LH$_2$ is required, L-LH$_2$ is inactive for light emission. Reaction [2] is the oxidative decarboxylation of the bound luciferyl adenylate and produces an enzyme-product complex, CO_2, AMP, and light. When substrates are present at saturating concentrations, the enzyme is strongly inhibited by the product, oxyluciferin, and normally turns over only twice (2). In addition to the above reactions, luciferase will also catalyze reaction [3] the activation of the competitive inhibitor dehydroluciferin (L) to form a tightly bound complex, E · LAMP.

$$\text{L} + \text{ATP} + \text{E} \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{E}\cdot\text{L-AMP} + PP_i \quad [3]$$

Since dehydroluciferin cannot be oxidized the reaction stops at E · L-AMP.

High concentrations of AMP, PP_i, and anions such as SO_4^{2-} are inhibitors of the enzyme (3). Prior to this study there were no examples of substances that would

[1] This research was supported in part by a grant from the National Science Foundation, PCM78-266221-A02 to Dr. Marlene DeLuca.

[2] The award of a Travelling Fellowship by the Medical Research Council (London) to Dr. Larry J. Kricka is gratefully acknowledged.

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[5] Abbreviations used: E, firefly luciferase enzyme; L, dehydroluciferin; LH$_2$, luciferin; Tricine, *N*-tris(hydroxymethyl)-methyl glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; BSA, bovine serum albumin; CMC, critical micelle concentration.

0003-9861/82/100674-08$02.00/0

stimulate enzymatic activity. In this paper we present results of the effects of various solvents on the catalytic activity of luciferase. Enzymatic activity was stimulated by these solvents up to sevenfold.

MATERIALS AND METHODS

Glycylglycine, Tricine, and Hepes were obtained from Sigma Chemical Company, St. Louis, Missouri 63178. Adenosine 5'-triphosphate and bovine serum albumin (A grade) were obtained from Calbiochem-Behring Corporation (La Jolla, Calif. 92122). Luciferin and dehydroluciferin were synthesized as described previously (4). Firefly luciferase was purified according to the procedure of Green and McElroy (1). Bacterial luciferase was purified from a frozen cell paste of *Beneckea harveyi*, and was assayed as described previously (5). Inorganic[^{32}P]pyrophosphate, specific activity 20 mC/μmol, was purchased from New England Nuclear, Boston, Massachusetts 02118.

Solvents

Nonionic. Polyethylene glycol (PEG, approximate molecular weights 600 and 8000), polyvinylpyrrolidone (PVP, average molecular weight 40,000), dextran (clinical grade, average molecular weight 173,000), and polyoxyethylene sorbitan monolaurate (Tween 20), were purchased from Sigma Chemical Company. PEG (Aquacide III; average molecular weight 20,000) was obtained from Calbiochem-Behring Corporation. Ethylene glycol, propane-1,2-diol, and glycerol were supplied by Mallinckrodt Inc., Paris, Kentucky 40361. Triton X-100 (average molecular weight 646) was obtained from A. J. Lynch and Company, Los Angeles, California 90058. Lauryl maltoside and octyl glucoside were gifts from Professor Shelagh Ferguson-Miller (Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823).

A sample of pure PEG 8000 (without added antioxidants) was kindly supplied by Dr. Louis F. Theiling, Union Carbide Corporation, South Charleston, West Virginia.

Cationic. Cetyltrimethyl ammonium bromide was purchased from Sigma Chemical Company.

Anionic. Sodium lauryl sulfate and sodium deoxycholate were obtained from Sigma Chemical Company.

Zwitterionic. Zwittergent 3-14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) was purchased from Calbiochem-Behring Corporation.

Firefly luciferase assay. The stock assay mixture consisted of 8 ml glycylglycine buffer (0.025 M, pH 7.8), 0.5 ml magnesium sulfate (0.1 M, pH 7.8), and 0.8 ml luciferin (10^{-3} M). Luciferase (10 μl, 80 pmol/ml) was added to 400 μl of assay mixture, and the reaction was initiated by injection of 100 μl of 0.02 M ATP. The peak light intensity was recorded with an Aminco Chem-Glow photometer (American Instruments Company, Silver Springs, Md. 20910). Total light emitted was calculated from the area under the light-time curve.

Investigation of Effect of Different Solvents on the Bioluminescent Firefly Luciferase Reaction

Solvents were prepared in phosphate buffer (0.1 M, pH 7.0). The pH of the solutions was adjusted to pH 7.0 using either sodium hydroxide or hydrochloric acid.

The solvent (50 μl) or as a control, 0.1 M phosphate buffer, pH 7.0 (50 μl), was added directly to the assay mixture (350 μl). The enzyme was then added to this mixture and luciferase activity determined as described previously.

In order to achieve very high solvent concentrations in the assay mixture, the solvents were made up to the appropriate concentration in the glycylglycine buffer and used instead of this buffer in the assay mixture. A range of concentrations of the following solvents was studied: PEG 600 (0-50 g/liter), PEG 8000 (0-50 g/liter) PEG 20,000 (0-25 g/liter), PVP (0-20 g/liter) dextran (0-10 g/liter) Triton X-100 (0-16 mM), Tween 20 (0-8.2 mM), ethylene glycol (0-0.8 M), propane-1,2-diol (0-0.66 M), glycerol (0-0.54 M), sodium deoxycholate (0-1 mM), sodium lauryl sulfate (0-3.5 mM), cetyl trimethyl ammonium bromide (0-6.9 mM), lauryl maltoside (0-10 mM), octyl glucoside (0-20 mM), and Zwittergent 3-14 (0-1 mM).

Spectra of emitted light. The spectrum of the light emitted by firefly luciferase reaction in the presence of PVP, PEG, and Triton was measured using a Spectrofluorometer Mark I (Farrand Optical Co., Inc., NY). The reaction was initiated by adding ATP and allowed to proceed for 30 s until the light output was constant; then the emission spectrum was measured.

^{32}P-Inorganic pyrophosphate exchange. $^{32}PP_i$ exchange into ATP was measured as a function of time in the presence of 1×10^{-4} M dehydroluciferin and 5 μg of enzyme. The assay conditions were the same as described by Mehler and Stern (6).

L-AMP titration. The number of binding sites for L-AMP on the enzyme can be determined by measuring the fluorescence of dehydroluciferin in the presence of enzyme and ATP-Mg^{2+}. The enzyme bound L-AMP is essentially nonfluorescent whereas free L is fluorescent (7).

Aliquots of L are added to the enzyme and ATP-Mg^{2+} and the fluorescence is monitored after each addition. When all of the sites are saturated with L-AMP, further addition of L results in a marked increase in fluorescence. In a typical experiment 4×10^{-6} M luciferase in 0.025 M glycylglycine, pH 7.8,

containing 2×10^{-3} M ATP and 5×10^{-3} M $MgSO_4$ was mixed. L was added in 10-μl increments and the fluorescence measured after each addition. Each addition increased the concentration of L in the cuvette by 5×10^{-7} M. Fluorescence was measured on a Perkin-Elmer MPF-44A Spectrofluorometer with an exciting wavelength of 358 nm and emission at 551 nm.

Effect of protein on solvent stimulation of the firefly luciferase reaction. Luciferase assay buffer (350 μl), 50 μl of PEG 8000 (250 g/liter), 5, 10, 25, 50, or 100 μl of BSA (80 mg/ml) in phosphate buffer (0.015 M, pH 7.4) containing sodium chloride (3 g/liter), and 10 μl of firefly luciferase in phosphate buffer (0.1 M, pH 7.0) were placed in an assay tube. The total volume was adjusted to 500 μl by addition of phosphate buffer (0.1 M, pH 7.0). A 100-μl aliquot of ATP (0.02 M, pH 7.0) was then injected and the peak light emission measured. Similar experiments were performed with 10 and 40 mg/ml BSA solutions. The effect of BSA on the PVP-, Triton-, lauryl maltoside-, and Zwittergent-stimulated firefly reaction was investigated in a similar fashion.

Polyacrylamide disc gel electrophoresis. This was carried out in 7.5% gels in 0.05 M phosphate buffer, pH 8.0, for 4 h at 80 V. Samples of luciferase (10 μg) and as a control catalase (10 μg) were electrophoresed in the presence and in the absence of Triton (2 mM), PEG 20,000 (25 g/liter) and PVP (5 g/liter).

RESULTS

Table I summarizes the data obtained with various solvents. Stimulation or inhibition of firefly luciferase activity was dependent on the concentration of the solvent in all cases.

Figure 1 illustrates the concentration dependence of the stimulation by various

TABLE I

EFFECTS OF SOLVENTS ON THE CATALYTIC ACTIVITY OF FIREFLY LUCIFERASE

	Optimal final concentration	Relative activity based on peak light emission (% control)
Solvent		
A. Nonionic		
Ethylene glycol	0.8 M	200
Propane-1,2-diol	0.66 M	200
Glycerol	0.54 M	293
PEG 600	50 g/liter (83 mM)[a]	320
PEG 8000	50 g/liter (6.25 mM)[a]	327
PEG 20,000	25 g/liter (1.15 mM)[a]	382
PVP 40,000	5 g/liter (0.115 mM)[a]	640
Dextran 173,000	10 g/liter (58 μM)	136
Micelle-forming solvents		
B. Nonionic		
Triton X-100	0.75 mM	500
Tween 20	0.82 mM	450
Lauryl maltoside	0.5 mM	545
Octyl glucoside	9 mM	200
C. Cationic		
Cetyltrimethyl ammonium bromide	6.9 mM	−100[b]
D. Anionic		
Lauryl sulfate	3.5 mM	−100
Deoxycholate	1 mM	−100
E. Zwitterionic		
Zwittergent 3-14	1 mM	544

[a] Molar concentration based on the average molecular weight.
[b] Concentration at which complete inhibition was observed.

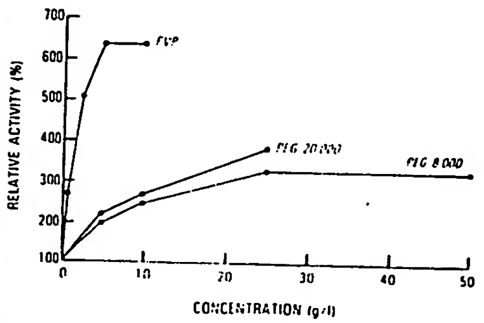

FIG. 1. Concentration dependence of stimulation of peak light emission of a firefly luciferase reaction by PVP, PEG 8000, and PEG 20,000. Concentration shown is final concentration in assay tube.

nonionic substances (Group A, Table I). Stimulation was observed in a variety of buffers (glycylglycine, phosphate, Hepes, Tricine). PEG stimulation increased with increasing average molecular weight of the PEG. At very high PEG 8000 concentrations (200 g/liter) a diminution in the stimulation was observed. Among the substances in Group A (Table I), PVP produced the largest stimulation of the luciferase reaction. In the presence of PVP or PEG a marked foaming was noticed in the spent assay mixture. A control experiment using bacterial luciferase in the presence or absence of PEG or PVP revealed that the foaming did not impair light detection. Light production by this enzyme was the same with or without PEG.

The concentration-dependent stimulation of the luciferase reaction by nonionic micelle-forming substances (Group B, Table I) was markedly different from that observed with substances which do not form micelles (Group A). For example, at low Triton concentrations no stimulation was observed, but above a certain critical Triton concentration a sudden and dramatic stimulation occurred (Fig. 2). A similar concentration dependence of the stimulation was observed with the other nonionic surfactants investigated (Tween 20, lauryl maltoside, octyl glucoside).

Anionic and cationic surfactants (Groups C and D, Table I) completely inhibited the luciferase reaction at high concentrations. Inhibition diminished as the concentration of these substances in the assay buffer was progressively lowered.

In contrast to the anionic and cationic surfactants the zwitterionic surfactant "Zwittergent" (Group E, Table I) stimulated luciferase activity and the stimulation showed a concentration dependence similar to that observed with the nonionic surfactants (Group B).

Different Firefly Luciferase Preparations

In order to preclude the observed stimulation being an artifact of the particular firefly luciferase preparation, two other preparations were tested. PEG gave comparable stimulations with both of these preparations.

Wavelength of the Emitted Light

The wavelength maxima of the emitted light of the luciferase reaction in the presence or absence of various solvents were identical within experimental error and agreed with the previously published value of 560 nm (8).

Kinetics

The only apparent differences in the kinetics of light emission were observed in the presence of Triton and Tween. Figures 3A, B, and C show the time course of light emission from a control, a Triton-stimu-

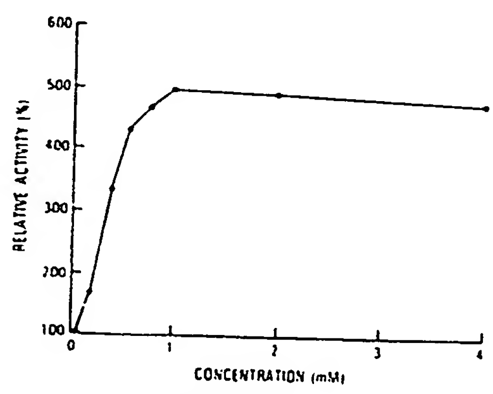

FIG. 2. Concentration dependence of stimulation of peak light emission of a firefly luciferase reaction by Triton X-100. Concentration shown is final concentration in assay tube.

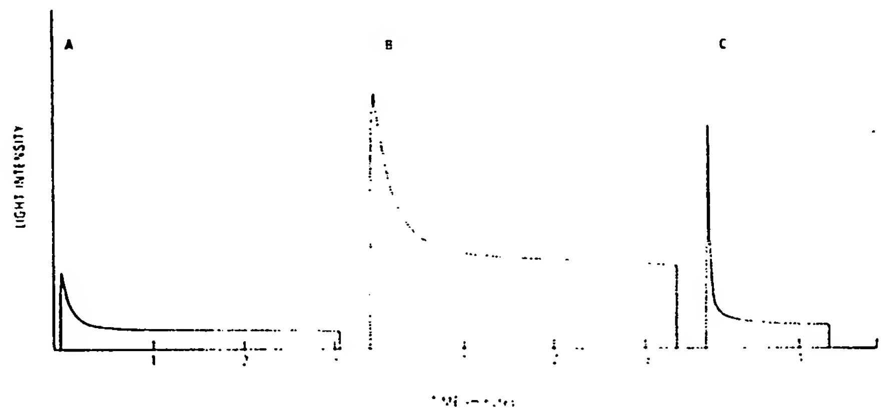

FIG. 3. Kinetics of firefly luciferase bioluminescence. (A) control; (B) in the presence of Triton (0.05 mM); and (C) in the presence of Tween (0.75 mM). Enzyme concentrations were identical in all three experiments and the recorded light intensities are directly comparable.

lated, and a Tween-stimulated reaction, respectively. Flash height was increased by Triton and by Tween, and for the latter, the time to reach peak light was more rapid than was observed with the control (Fig. 3). The decay of light emission, which reflects the rate of product inhibition, from the Triton-stimulated reaction was significantly slower than that of the control (Fig. 3A vs B). Measurement of the total light emitted during a three-minute period revealed a comparable stimulation in peak and total light emission for both the Triton and Tween-stimulated reactions.

Order of Addition of Substrates

If luciferin or luciferase is injected to initiate the reaction rather than ATP, then the Triton stimulation of peak light emission was abolished but total light emission was increased twofold. Again the decay rate was slower than that of the control.

Injections of solutions of Triton or PEG after the initial flash of light caused a slow increase in light output which peaked and then slowly decayed (Fig. 4). A similar increase in light ouput was observed with PEG.

Mechanistic Studies

The Michaelis-Menten constants (K_m) for luciferin and for ATP-Mg^{2+} in the presence of PEG and in the presence of Triton were unchanged from values obtained in the absence of these solvents. In order to determine if the Triton and PEG were unmasking additional active sites on the enzyme, titration of the active sites with L and ATP-Mg^{2+} was done in the presence and absence of these substances. In all cases one L-AMP was formed per 100,000 daltons of enzyme in agreement with previous results (9).

Another measure of the activation reaction, Eq. [1], can be determined from the rate of exchange of $^{32}PP_i$ into ATP in the presence of L. Triton caused a small (30%) stimulation of the exchange rate; however, this is not nearly as great as the observed enhancement of light emission.

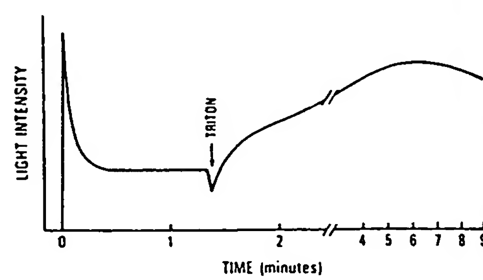

FIG. 4. Effect of a postflash injection of Triton on the kinetics of the firefly luciferase reaction. At 0 time ATP was injected into a standard luciferase assay mixture. At the point indicated, 50 µl of 4 mM Triton was injected.

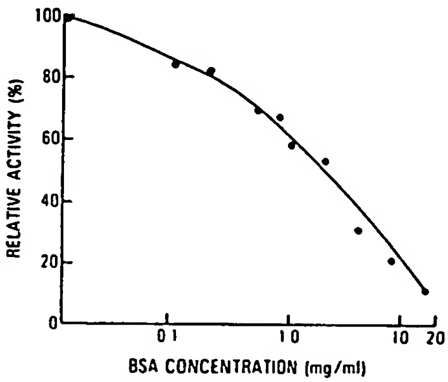

FIG. 5. Effect of BSA on PEG 8000-stimulated firefly bioluminescence. Concentration shown is final concentration in assay tube. Assay conditions are described under Materials and Methods.

Protein Effects

BSA (4 mg/ml in assay mixture) produced a twofold stimulation of peak light emission. However, BSA produced a concentration-dependent reduction in the PEG, PVP, Triton, lauryl maltoside, and Zwittergent stimulation of the luciferase reaction (e.g., Fig. 5).

Gel Electrophoresis

When luciferase is electrophoresed in 7.5% acrylamide gels in phosphate buffer, between four and five bands are seen, presumably due to aggregation of the enzyme. If the gels are run in the presence of Triton, none of the enzyme moves into the gels. Catalase, M_r 232,000 daltons, migrates into the gel in the presence of Triton. Apparently the Triton and luciferase form a large complex which is excluded from these gels. In gels containing PVP or PEG the migration of luciferase and the control (catalase) was also reduced.

DISCUSSION

The bioluminescence of the firefly luciferase reaction is stimulated by a variety of substances with differing molecular weights and chemical properties. Generally, nonionic surfactants are the most effective stimulants for the reaction and for these substances the most probable mechanism is one involving micelles. The relationship between stimulation and surfactant concentration shows the characteristic abrupt change in stimulation above a certain critical surfactant concentration which approximated to the critical micelle concentration (CMC). The observed CMC's based on stimulation of luciferase activity were—Triton X-100, 0.75 mM; Tween 20, 0.82 mM; laurylmaltoside, 0.05 mM; octyl glucoside, 9 mM: literature values are 0.12, 0.24, 0.2, and 20 mM (10), respectively.

PEG, which does not form micelles, and Triton, which does form micelles, were chosen for extensive investigation. Both of these stimulate light production between 1.5- and 5-fold. The stimulation is not due to an unmasking of additional active sites as demonstrated by the titration with L and ATP-Mg' in the presence or absence of the polymers. It is not due to a change in K_m's for the substrates. It is not likely that either PEG or Triton are concentrating LH_2 near the active site of the enzyme, since LH_2 is present in saturating concentrations in the experiments. The oxidative reaction is effected since there was very little increased activity in the $^{32}PP_i$-ATP exchange reaction. While the exact mechanism of the stimulation is unknown, there must be increased turnover of the enzyme in the presence of these solvents. This is apparent from the increased total light obtained and also since the quantum yield of the reaction is 0.88 it is not possible that the stimulation is due to an increased quantum yield (11). The stimulation of luciferase by PEG and Triton after the initial flash of light (Fig. 4) when the enzyme is product inhibited is compelling evidence that both PEG and Triton are increasing the turnover of the enzyme. One reasonable explanation is that the polymers bind to the enzyme in such a manner that the product molecule, which is inhibitory and normally tightly bound, is somehow more readily dissociated.

An interesting observation is that Triton does not stimulate peak height if the enzyme reaction is initiated by injecting LH_2 or enzyme. However, in both cases the total light was increased. When enzyme

is mixed with LH_2 and ATP injected, both peak light and total light are stimulated. The nonequivalence of the results when LH_2, ATP, or enzyme initiates the reaction suggests that interactions between Triton micelles and the enzyme and substrates are different and either irreversible or only slowly reversible in the heterogeneous reaction mixture. Most light is obtained from reactions in which Triton and LH_2 are mixed together, i.e., enzyme or ATP injection. Results from two-phase water/Triton experiments show that LH_2 distributes preferentially into the Triton phase. Thus interaction between enzyme and LH_2 contained in Triton micelles may be a key event in the stimulation of light output. The enzyme is known to undergo a large conformational change during reaction (12) and it is possible that the Triton-LH_2 enzyme interaction promotes this conformational change. There are several reports of binding of Triton X-100 to proteins (13) and the inhibition of stimulation by BSA suggests that BSA may compete with the enzyme for binding to Triton, thus preventing the stimulation.

The mechanism proposed for the Triton stimulation cannot operate for substances such as PEG, which does not form micelles. Although it has been shown that PEG does not bind to certain soluble proteins (14), firefly luciferase is a very hydrophobic protein and thus may be an exception (7). Thus luciferase:PEG aggregates may act in a similar manner to luciferase:Triton aggregates. Evidence from gel electrophoresis supports this hypothesis. In the presence of PEG and also in the presence of PVP, electrophoresis was impaired, which suggests the formation of high molecular weight complexes. However, an alternative explanation is that in the presence of PEG the enzyme precipitates at the top of the gel (15).

Anionic and cationic surfactants failed to stimulate the luciferase reaction. The reasons for this are obscure since positively charged micelles formed from cationic surfactants would be expected to stimulate the luciferase reaction, since all of the substrates are negatively charged.

Stimulation by the lower molecular weight substance, e.g., ethylene glycol and glycerol, is difficult to explain. Both are widely used to stabilize enzyme (16) but we are unaware of any reports of stimulations of enzyme turnover.

The rates of many organic reactions are altered by surfactants. This type of stimulation is normally explained in terms of solubilization and concentration of reactants by micelles (17). Studies of enzyme-catalyzed reactions in the presence of surfactants is less extensive (18). Both increased and decreased rates of reaction have been observed depending upon the particular enzyme and surfactant. For example, Triton X-100 stimulates the activity of glucose-6-phosphate phosphohydrolase; whereas sodium lauryl sulfate and Tween 20 inhibit the activity of this enzyme (19). An apparent stimulation of a lactate dehydrogenase-mediated reduction of tetrazolium salts by Triton X-100 has also been described (20). No detailed mechanistic interpretations or generalizations have been proposed to explain micelle effects on enzyme catalyzed reactions. In the solvent-stimulated luciferase reactions described here, solvents such as Triton and PEG stimulate enzyme activity by increasing the turnover of the enzyme but as yet the exact mechanism of this stimulation remains unknown.

ACKNOWLEDGMENTS

We thank Steve Alter for performing the gel electrophoresis experiments and Dr. Clarence Suelter for helpful discussions.

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